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14. ABSTRACT

Prostate cancer is a major contributor to cancer mortality in U.S. men. Prostate cancer mortality results from metastasis and progression from an androgen-dependent to an androgen-independent disease. Proteins on the cell surface are critical to the tumor-microenvironment interactions. This project focuses on identifying the disrupted cell surface proteins by proteomic approaches, determining their functional significance in prostate cancer progression and metastasis, and evaluating their usage as diagnostic and prognostic markers for prostate cancer. We performed quantitative proteomic analysis of multiple prostate cell lines with different tumorigenic and metastatic potentials. We have published in-depth studies how a cell surface protein EMMPRIN promotes prostate cancer cell migration and metastasis. We also found that the level of the cell surface protein EMMPRIN progressively increased with increasing age of TRAMP mice (6-27 wks); with the highest detected in liver metastases. Quantitative analysis revealed that by 27-wks (an age exhibiting highly aggressive prostate tumor phenotype), EMMPRIN expression increased significantly (P=0.001). The results suggest that EMMPRIN may have diagnostic value in prostate cancer detection in advanced disease.

15. SUBJECT TERMS
Prostate cancer, cell surface proteins, proteomics, EMMPRIN, cell migration, metastasis, biomarkers

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Biomarker Discovery and Mechanistic Studies of Prostate Cancer using Targeted Proteomic Approaches

W81XWH-08-1-0430

Initiating PI: Haining Zhu

Partnering-PI: Natasha Kyprianou

Introduction

The focus of this collaborative work between Drs. Zhu and Kyprianou has been the identification of EMMPRIN, a cell surface protein we identified to be overexpressed in prostate cancer epithelial cells with a high metastatic potential. Our studies in the funding period have identified the involvement of EMMPRIN in cancer development and progression via controlling extracellular matrix remodeling and anchor-independent growth by stimulating MMP production, angiogenesis via VEGF by activation of AKT-PIK3 pathway, and cell invasion by up-regulation of urokinase-type palsminogen activator. The emerging theme of pursuing the role of EMMPRIN as a functional biomarker in prostate cancer metastasis is being tested in the last year of the funding period. The results have also led us to determine the status of additional proteins that control the actin-cytoskeleton organization such as cofilin.

Body

Targeting of tumor cell metastasis is of major therapeutic significance and its exploitation may lead to the identification of effective new modulations such as : (**1**) reversing the ability of tumor cells of becoming resistant to anoikis,

therefore making them more susceptible to anoikis-inducing agents; (**2**) interfering with the seeding process of tumor cells into secondary places by making tumor cells non-sensitive to the chemotatic and environmental cues of the new target organ; and (**3**) making these secondary targets less “appealing” to the cancer cells by blocking key molecules promoting cancer cell seeding and survival. Membrane proteins play a critical role during the metastasis process since they regulate cell-cell interactions and coordinate cell-tumorenvironment communication. The initiating PI supported by this PCRP Synergistic Grant (USAMRMC PC074317) Dr. Zhu utilized the proteomic approaches and identified EMMPRIN as one of differentially expressed membrane proteins in prostate cells. Significantly higher levels of EMMPRIN protein were detected in highly metastatic human prostate cancer cells. Subsequent validation studies in Dr. Kyprianou’s lab led to the identification of additional proteins that regulate the cytoskeleton organization as potential regulators of prostate cell migration, cell-cell interactions and ultimately invasion and metastasis.

Task 1. The current focus is on a) the mechanistic dissection of EMMPRIN’s contribution to metastasis and b) the significance of EMMPRIN in human prostate cancer progression to metastastic disease and clinical outcomes to define the potential value of this player as a marker of metastasis, studies will pursue expression profiling of EMMPRIN proteein levels in a series of human prostate cancer specimens of increasing Gleason grade and metastastic lesions. Human prostate tissue specimens from patients with primary and metastatic prostate tumors (Department of Pathology, University of Pittsburgh), were subjected to immuno-profiling for EMMPRIN expression and quantitative analysis will be achieved using computer-image analysis in normal prostate; benign prostate hyperplasia, BPH; prostate primary tumors (Gleason Score range 6-9); and metastatic lesions (n=45). Ongoing translational studies focus on establishing a correlation between EMMPRIN expression with serum PSA levels, Gleason grade and patient (disease-free) survival in a large cohort of

prostate cancer patients, which may define the value of EMMPRIN as a cancer metastasis marker.

Task 2. Ongoing experiments investigate the expression of a critical tight junction protein, ZO-1 in prostate tumors with increasing grade. Preliminary staining revealed clear striations of Tight Junctions visualized in epithelial regions that are strongly detected in low-grade tumors and expression is decreased with increasing age of the TRAMP mice. Prostate tumors from 20, 24, 27 and 31-week-old mice are currently being interrogated for tight junction protein expression that will be correlated with the EMMPRIN expression (an inverse correlation is expected). Please see attachment.

Task 3. Experiments will be pursuing the consequences of EMMPRIN loss/silencing in prostate cancer cells on the transcriptional regulation of the major players of the process of Epithelial Mesenchymal Translation (EMT). The prostate tumor microenvironment represents a key component of the invasive dynamic of prostate cancer. This new exciting direction of the work is directly developed from the studies supported by this program.

Comprehensive Presentation of Results

Development of metastatic prostate cancer is orchestrated by multiple signaling pathways that regulate cell survival, apoptosis, epithelial-mesenchymal transition (EMT), invasion, cytoskeleton remodeling/signaling and angiogenesis. Disruption of the mechanisms underlying these processes and the phenotypic characteristics of their manifestation is critical for metastasis and enhancing therapeutic sensitivity of metastatic tumors to anti-angiogenesis strategies. Targeting the AKT survival pathway and preventing angiogenesis by reducing tumor cell adhesion to the extracellular matrix (ECM), blocking actin organization and filopdia formation and impairing metastasis. Building on the recently published evidence on the functional involvement of

EMMPRIN to regulate cytoskeleton reorganization and impact prostate cancer cell invasion (*The Prostate*, 72(1):72-81, 2012), we subsequently pursued the mechanistic dissection of actin remodelling towards promotingprostate tumor progression to metastasis. The actin depolymerizing factor (ADF) cofilin, a small (19kDa) actin binding protein was previously identified by our group to be an intracellular effector of transforming growth factor beta (TGF-β) in prostate cancer cells. Directing our investigative efforts to identify the consequences of a mutation in cofilin phoshorylation site Serine 3 residue; (S3ACFL) we used the human prostate cancer cell line, that is TGF-β sensitive, androgen-independent PC-3 cells as model. This study has been recently submitted for publication. The results are intriguing as they demonstrated a significant increase in the migration ability for S3ACFL prostate cancer cells compared to the wild type controls. Furthermore there was enhanced adhesion to fibronectin in S3ACFL PC-3 cells, potentially driven by the significantly higher number of filopodia structures in cells harboring the cofilin mutation. TGF-β treatment decreased cell migration, adhesion and filopodia protrusions in the cofilin S3A cells. Of major translational significance that becomes a key accomplishment for this work Is the immunohistochemical analysis of human prostate cancer specimens from primary prostate tumors and lymph node metastatic lesions for expression of cofilin, p-cofilin, palladin and e-cadherin. These proteins are critical players in actin-remodeling through actin cytoskeleton organization and control of epithelial-mesenchymal-transition. Cofilin immunoreactivity correlates with higher tumor grades and moreover there was a significant upregulation of cofilin in metastatic lesions compared to primary tumors. *In vivo* characterization of the metastatic potential of WTCFL and S3ACFL PC-3 cells revealed an increased number of lung metastatic lesions due to cofilin mutation.

The novel findings gathered through this synergy of multidisciplinary approaches, provide evidence to support the ability of cytoskeleton organization regulator proteins, EMMPRIN and cofilin, promote cytoskeletal reorganization

of prostate cancer metastasis and establish for the first time the predictive value of both proteins in prostate cancer progression to metastasis.

Key Research Accomplishments

This work represented a most creative interaction between two investigators with complementary expertise, prostate tumor biology and proteomics technology with powerful precision towards functional assessment of the biological relevant contributions at both the mechanistic and translational level. Through the proteomic analysis of human prostate cancer cells with different invasive characteristics and metastatic potential, the award enabled us to a) identify two novel markers of metastatic progression and aggressive phenotype of human prostate cancer; and b) to mechanistically dissect their functional role in the organization of actin cytoskeleton dynamics and remodeling, a fundamentally critical role that heavily impacts the metastastic behavior of prostate cancer cells. The two key proteins that emerged from these synergistic studies, cofilin and EMMPRIN may prove potent and valuable biomarkers of prostate cancer progression in the clinical setting of prostate cancer patients.

Our collaborative studies showed that EMMPRIN loss in human prostate cancer cells had no significant consequences on prostate cell growth, proliferation or apoptosis. However, we found a significant suppression in prostate tumor cell invasion, migration and metastatic ability using in vitro assays. These data are reported in the manuscript to be published in the Prostate (please see as Appendix 1, a copy of the manuscript by Zhu et al, *The Prostate*, 72(1):72-81, 2012) .

Our collaboration at the translational level has been the determination of the potential predictive value of EMMPRIN in prostate cancer progression, first utilizing the TRAMP mouse model of prostate tumorigenesis and subsequently analyzing a series of human prostate cancer specimens of increasing Gleason grade. The TRAMP mice (C57BL/6J) are transgenic mice that express SV40T/t antigens under the prostate specific rat probasin promoter. TRAMP transgenic

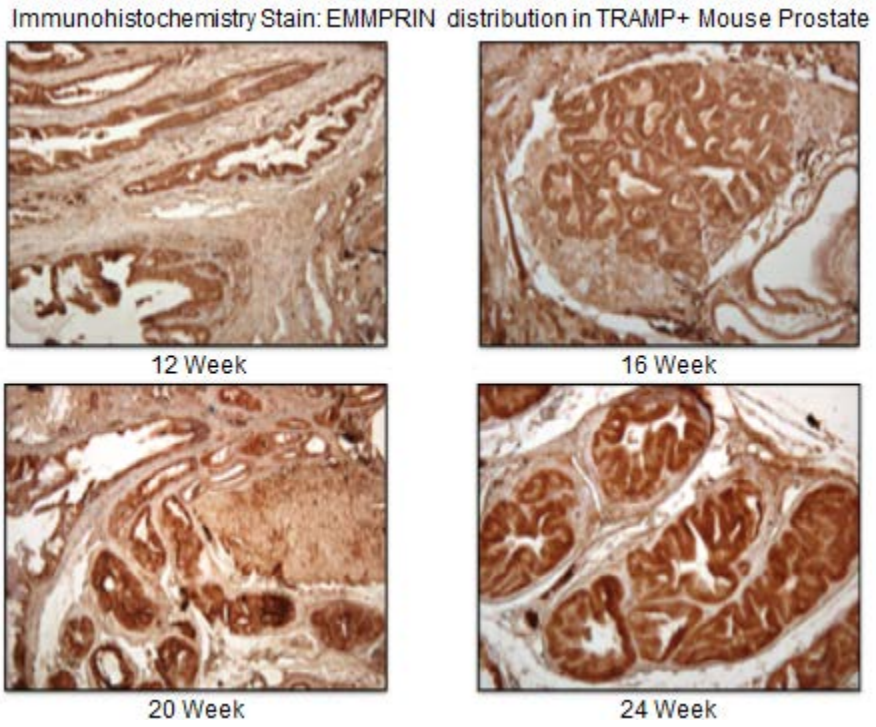
males develop prostate adenocarcinoma in a manner resembling the clinical progression of human prostate cancer from intra-epithelial neoplasia to androgen-independent metastatic tumors. Hematoxylin and eosin (H&E)-stained sections of prostate tissues from TRAMP/+/+ male mice were evaluated to confirm pathological grade. Prostate sections from wild type and the TRAMP tumors of increasing grade and metastatic lesions (5μm) were subjected to immunohistochemical analysis for EMMPRIN expression. As shown in Figure 1 histopathological grading of prostatic tumors revealed that in the majority of 16-24wk-old TRAMP mice, prostate adenocarcinoma was evident (16-20weeks), and with increasing age (24 weeks), poorly differentiated tumor foci were detected with focal cribriform lesions protruding into the lumen (grade 3-5), representing tumor progression to advanced disease. A score for each histological grade (H) was determined as the product of intensity and proportion ($H = I \times P$).

Reportable Outcomes

Three collaborative publications result from the studies supported by the Synergistic Idea Development Award.

1. Tang, X., Tang, X., Gal, J., **Kyprianou, N**., **Zhu, H.** and Tang, G. Detection of microRNAs in prostate cancer cells by microRNA array. Methods in Molecular Biology: MicroRNAs in Development, 732:69-88, 2011.
2. **Zhu, H.,** Zhao, J., Zhu, B., Collazo, J., Gal, J., Shi, P., Liu, L., Strom, A.L., Lu, X., McCann, R.O., Toborek, M., and **Kyprianou, N**. EMMPRIN Regulates Cytoskeleton Reorganization and Prostate Cancer Cell Invasion, *The Prostate*, 72(1):72-81, 2012. (Appendix 1).
3. Martin, S.K., Vaughan, T.B., Atkinson, T., **Zhu, H.** and **Kyprianou, N**. Prostate Cancer Biomarker Update*. Oncology Reports,* 28: 409-417*,* 2012. (Appendix 2)

Figure 1


Immunohistochemistry Stain: EMMPRIN distribution in TRAMP+ Mouse Prostate
12 Week
16 Week
20 Week
24 Week

Appendix 1

Zhu, H., Zhao, J., Zhu, B., Collazo, J., Gal, J., Shi, P., Liu, L., Strom, A.L., Lu, X., McCann, R.O., Toborek, M., and **Kyprianou, N**. EMMPRIN Regulates Cytoskeleton Reorganization and Prostate Cancer Cell Invasion, *The Prostate*, 72(1):72-81, 2012.

EMMPRIN Regulates Cytoskeleton Reorganization and Cell Adhesion in Prostate Cancer

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BACKGROUND. Proteins on cell surface play important roles during cancer progression and metastasis via their ability to mediate cell-to-cell interactions and navigate the communication between cells and the microenvironment.
METHODS. In this study a targeted proteomic analysis was conducted to identify the differential expression of cell surface proteins in human benign (BPH-1) versus malignant (LNCaP and PC-3) prostate epithelial cells. We identified EMMPRIN (extracellular matrix metalloproteinase inducer) as a key candidate and shRNA functional approaches were subsequently applied to determine the role of EMMPRIN in prostate cancer cell adhesion, migration, invasion as well as cytoskeleton organization.
RESULTS. EMMPRIN was found to be highly expressed on the surface of prostate cancer cells compared to BPH-1 cells, consistent with a correlation between elevated EMMPRIN and metastasis found in other tumors. No significant changes in cell proliferation, cell cycle progression, or apoptosis were detected in EMMPRIN knockdown cells compared to the scramble controls. Furthermore, EMMPRIN silencing markedly decreased the ability of PC-3 cells to form filopodia, a critical feature of invasive behavior, while it increased expression of cell–cell adhesion and gap junction proteins.
CONCLUSIONS. Our results suggest that EMMPRIN regulates cell adhesion, invasion, and cytoskeleton reorganization in prostate cancer cells. This study identifies a new function for EMMPRIN as a contributor to prostate cancer cell–cell communication and cytoskeleton changes towards metastatic spread, and suggests its potential value as a marker of prostate cancer progression to metastasis. *Prostate 72: 72–81, 2012.*

KEY WORDS: **prostate cancer; EMMPRIN; cytoskeleton; shRNA; filopodia**

Additional supporting information may be found in the online version of this article.

Abbreviations: BPH, benign prostatic hyperplasia; ECM, extracellular matrix; EMMPRIN, extracellular matrix metalloprotease inducer; MMP, matrix metalloprotease; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium salt; PBS, phosphate buffer saline; SDS-PAGE, sodium dodecyl-sulphate polyacrylamide gel electrophoresis; shRNA, short hairpin RNA; TUNEL, terminal UTP end-labeling; VEGF, vascular endothelial growth factor.

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INTRODUCTION

Metastatic prostate cancer is a major contributor to cancer related mortality in men. Normal prostate epithelial cell homeostasis is maintained by a dynamic balance between cell proliferation and apoptosis. Normal cells undergo anoikis (a unique mode of apoptosis) upon detachment from extracellular matrix (ECM). Cancer cells however develop mechanisms to evade anoikis and acquire the ability to detach and migrate into new sites that provide a nurturing microenvironment for continued growth [1]. During the metastatic spread of primary tumor cells, proteins on cell surface are critical in mediating cell-to-cell and cell-to-environment communication.

EMMPRIN is a cell surface glycoprotein of IgG superfamily encoded by a gene localized to 19p13.3 [2,3]. EMMPRIN is an integral membrane protein, but may be released as a soluble protein by vesicle shedding [4,5]. It initiates the function through homophilic interactions between EMMPRIN molecules on neighboring cells [4,5]. EMMPRIN is expressed in numerous normal and malignant cells and mediates diverse processes such as angiogenesis, neuronal signaling, cell differentiation, wound healing, and embryo implantation [6]. Mice lacking EMMPRIN demonstrate various defects, including low embryonic survival, infertility, deficiencies in learning and memory, abnormality in odor reception, retinal dysfunction, and mixed lymphocyte reaction [6–10]. Elevated expression of EMMPRIN is found in several human cancers and correlates with the metastatic potential of tumor cells, specifically in breast and ovarian cancer epithelial cells during progression to metastasis [11–14]. In the context of the tumor microenvironment, EMMPRIN induces matrix metalloproteinase (MMP) production in stromal fibroblasts and endothelial cells as well as in tumor cells [11–13,15–17]. Elevated MMPs result in ECM degradation and subsequent detachment and metastasis of cancer cells. In addition, EMMPRIN can promote tumor cell invasion via activation of urokinase-type plasminogen activator [18], stimulate tumor angiogenesis by elevating vascular endothelial cell growth factor (VEGF) through Akt signaling [19], and causes multi-drug resistance in tumor cells via hyaluronan-mediated up-regulation and ErbB2 signaling activation [20]. EMMPRIN is implicated in metastasis via its ability to confer resistance of breast cancer cells to anoikis by inhibiting BIM [21], and its association with lipid raft or caveolae via interactions with key membrane proteins, including caveolin-1, monocarboxylate transporters, annexin II [22], and integrins $\alpha2\beta1$, $\alpha3\beta1$, $\alpha6\beta1$ [23], all critical in the spatial distribution and activity of EMMPRIN.

Previous studies suggested that EMMPRIN expression is associated with prostate cancer progression [24,25], and loss of EMMPRIN reduces the invasion potential of human prostate cancer cells [26]. This evidence however has been correlative and little is known about the mechanistic significance of EMMPRIN in prostate cancer progression and metastasis beyond its ability to induce MMPs. In this study we profiled the EMMPRIN expression pattern in human prostate cell lines of benign and metastatic origin and characterized the function of EMMPRIN in tumor cell aggressive behavior. EMMPRIN suppression led to a significant decrease in prostate cancer cell attachment to the ECM, migration and invasion, as well as filopodia formation while it enhanced cell–cell interactions. The results provide a new insight into the ability of EMMPRIN to regulate prostate cancer cell adhesion, invasion, and cytoskeleton organization.

MATERIALS AND METHODS

Cell Lines

The HEK293 and the human prostate cancer cell lines PC-3, DU-145, and LNCaP, were obtained from the American Type Culture Collection (Manassas, VA). The non-tumorigenic benign human prostatic epithelial cells BPH-1 (derived from human prostate epithelium of benign pathology) was generously provided by Dr. Simon W. Hayward (Department of Urological Surgery, Vanderbilt University Medical Center). Cells are maintained in RPMI-1640 medium (Gibco^{TM}, Grand Island, NY), supplemented with 10% fetal calf serum (CSS), 100 U penicillin and 100-mg/ml streptomycin, at 5% CO_2 incubator at 37°C.

Western Blot Analysis

Confluent cell cultures (80%) were washed with PBS, scraped, and cell pellets were harvested. Cells were disrupted with RIPA buffer (50 mM Tris-HCl, pH7.4, 1% NP40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mg/ml each of aprotinin, leupeptin, pepstatin, and 1 mM Na_3VO_4). Cell lysates were centrifuged at 5,000*g* (15 mins), resolved by SDS-PAGE, and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Upon incubation with the primary and secondary antibodies, immunoreactive bands were detected using a chemiluminescent approach with the ECL kit (Pierce, Rockford, IL). Membrane fractions were prepared using the protein isolation kit (Pierce). Monoclonal antibodies against EMMPRIN, ZO-2, actin, and tubulin were purchased from Santa Cruz Biotech (Santa Cruz, CA). Monoclonal antibodies against ZO-1 and AF6 were obtained from Invitrogen Zymed (San

Francisco, CA) and BD Transduction (Lexington, KY), respectively.

RT-PCR Analysis

Total RNA was extracted from cells using an RNAeasy kit (Qiagen, Valencia, CA). RNA samples (0.25 μg) were subjected to reverse transcription (RT) PCR reaction in a 20-μl volume with poly-oligoT primer. The resulting cDNA was subjected to PCR using EMMPRIN specific primers. The first set of primers started with exon 1 and ended at exon 11: EF2 (5′-ATG GCG GCT GCG CTG TTC GTG-3′) and ER11 (5′-GGA GCA GGG AGC GTC CTC GGG-3′). The second set of primers started with exon 2 and ended at exon 11: EF1 (5′-ATG AAG CAG TCG GAC GCG TCT C-3′) and ER11. GAPDH primers (5′-CAG CAA TGC ATC CTG CAC-3′ and 5′-GAG TTG CTG TTG AAG TCA CAG G-3′) were used as control in the same PCR reactions. Thirty cycles of PCR reactions were performed and each cycle included 45 sec, 94°C; 45 sec, 55°C; and 45 sec, 72°C. The PCR products were analyzed on a 1.2% agarose gel. Amplicons are purified, cloned, and sequenced by IDT (Coralville, IA).

shRNA Plasmid Construction and Transfection

Short hairpin RNA (shRNA) interference oligos, were designed using OligoEngine software (Seattle, WA) to specifically target EMMPRIN (NM_198589). Three oligos that target EMMPRIN (variant 2 mRNA) at nucleotides 98–116 (TGGCTCCAAGATACTCCTC), 277–295 (CCATGGGCACGGCCAACAT), and 776–794 (AGGCAAGAACGTCCGCCAG), are named as 98i, 277i, and 776i, respectively. A scramble shRNA (TTCTCCGAACGTGTCACGT) was used as control. The oligos are cloned to pSUPER (neo + GFP) plasmid from OligoEngine according to the manufacturer's instruction. Plasmids were amplified in DH5α cell and confirmed by sequencing.

Subconfluent cell populations were used for transfection using the FuGENE system (Roche, Indianapolis, IN). Briefly, the plasmid and Fugene reagent were combined and incubated for 20–30 min at room temperature. After transfection (36 hr), cells were subjected to cell sorting based on GFP expression and GFP positive cells were subsequently subjected to Western blotting. Stable tansfectants were cloned under Geneticin selection (Invitrogen) (300 μg/ml), the generated clones were maintained in RPMI 1640 medium (150 μg/ml Geneticin).

Cell Viability Assay

The MTT assay (based on the ability of viable mitochondria to convert MTT, a soluble tetrazolium salt, into an insoluble formazan precipitate) was used to assess cell viability. Cells were seeded into 96-well plates (2,500 cells/well) and incubated in growth medium (18–24 hr). After incubation with the MTT solution for 4 hr, absorbance was read at A_{570} and the colorimetric reaction product was quantitated spectrophotometrically (BioTek, PowerWave XS, Winooski, VT).

Evaluation of Cell Cycle and Apoptosis

BrdU/PI (Bromodeoxy uridine and propidium iodide) method was used for the analysis of cell cycle progression and apoptosis. Cells (1×10^6/ml) were incubated with BrdU (20 mM) (60 min at 37°C), suspended in PBS, and fixed with ice-cold 95% (v/v) ethanol. Fixed cells were subsequently permeabilized using pepsin (0.04% w/v, 0.4 mg/ml in 0.1 N HCl). BrdU was probed with FITC labeled anti-BrdU (BD, San Jose, CA). Apoptosis among the different cell populations was evaluated using the terminal UTP end-labeling (TUNEL) technique. (Leica, Germany).

Cell Adhesion Assay

The ability of cells to attach to key ECM components (fibronectin and laminin) was tested using fibronectin or laminin-coated 6-well multiwell plates (BD Bioscience). Prostate cancer epithelial cells were plated (10^5/well), and incubated at 37°C for 30 min, prior to fixing with methanol, and washed with PBS. Cells were counted from three random fields/well.

Evaluation of Cell Migration and Invasion

Confluent monolayer cells were wounded by scraping. Cultures were washed twice with medium, and then incubated at 37°C for 16 hr to allow migration toward the gap. The number of migrating cells was determined under the microscope. The invasion potential of prostate cancer cells was assessed using Biocoat Matrigel invasion chambers (Becton Dickinson). Briefly, cells (5×10^4) resuspended in RPMI1640-based medium were added (250 μl) into the invasion chambers and chambers were subsequently inserted into 24-well plates. Stained cells were photographed and counted.

Confocal Microscopy

Cells were plated on fibronectin-coated glass coverslips and fixed with 4% paraformaldehyde. Cells were permeablized in 0.1% (v/v) Triton-X 100 and were subsequently stained with rhodamine-phalloidin (Jackson ImmunoResearch, West Grove, PA). After rinsing with PBS (3×), slides were mounted with Vectorshield (Vector Lab, Burlingame, CA).

Slides were examined under a laser-scanning confocal microscope (Leica Lasertechnik, Heidelberg, Germany).

Cell Aggregation Assay

Cells aggregation assay was performed as previously described [27]. Briefly, cells were suspended into single cells and dissociated cells were allowed to associate in medium (1 hr) in 5% CO_2 at 37°C, with gentle rotation of the plates. The number of cell aggregates in the parental control PC-3 and EMMPRIN shRNA transfectant cells was counted.

Statistical Analysis

Data are expressed as mean ± SD. Mann–Whitney and Student's *t* tests were used to comparatively analyze the differences between groups in the various experiments.

RESULTS

EMMPRIN Expression in Human Prostate Cancer Cell Lines

Targeted proteomic analysis comparing the cell surface proteomes of BPH-1 (immortalized benign prostate hyperplasia cell line) and LNCaP and PC-3 (human prostate cancer cell lines derived from metastatic lesions) revealed the differential expression of EMMPRIN. EMMPRIN was found to be highly expressed on the cell surface of prostate cancer epithelial cells but not the benign prostate cells. Western blot analysis was subsequently conducted to validate the proteomics screening data and the results are shown in Figure 1. Using total cell lysates (Fig. 1, panel A), EMMPRIN showed a broad range molecular shift corresponding to different degrees of glycosylation as previously shown in breast cancer cells [11–13]. Malignant prostate cells, PC-3 and LNCaP appeared to have more highly glycosylated EMMPRIN than BPH-1 while the total protein levels were similar in all three cell lines. Glycosylation of EMMPRIN contributes to its membrane localization. Thus, plasma membrane fractions were isolated from all prostate cell lines and subjected to Western blotting. As shown in Figure 1B, EMMPRIN levels in the plasma membrane fractions of LNCaP and PC-3 cells were significantly higher than in BPH-1 cells. These results are consistent with the cell surface proteome studies, implicating higher EMMPRIN translocation to the plasma membrane in prostate cancer cells than in benign cells. The molecular mechanism of membrane targeting and translocation is beyond the scope of this article and is currently being pursued in a parallel study.

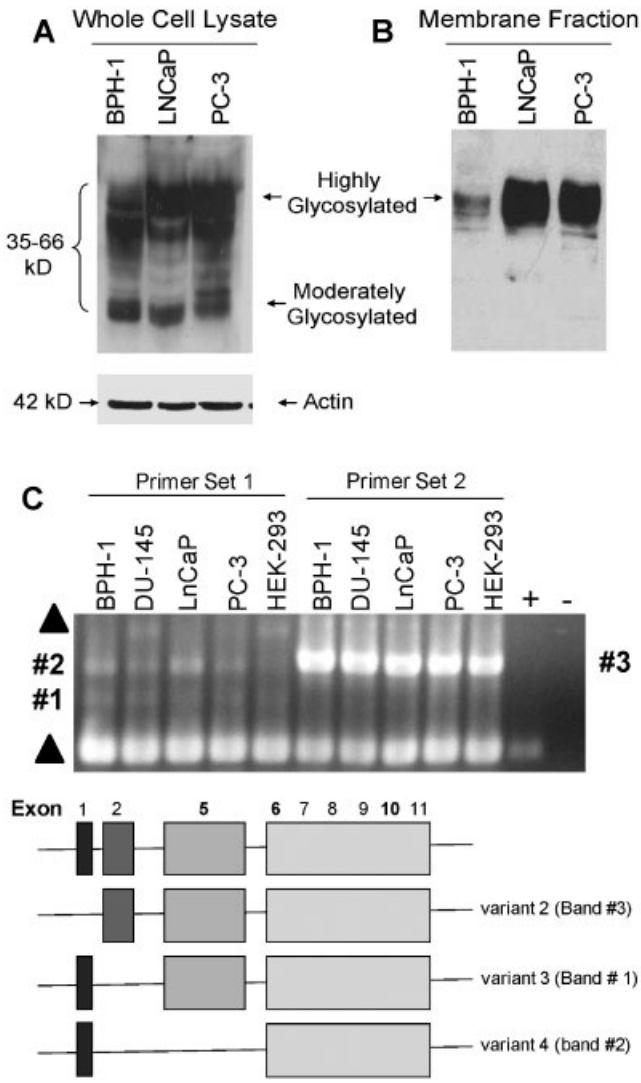

Fig. 1. EMMPRIN expression and alternative splicing in prostate cancer cells. Panel **A**: EMMPRIN expression levels in total cell lysates of BPH-1, LNCaP, and PC-3 cells. Bands of different motilities are likely due to glycosylation. Both LNCaP and PC-3 cell lines exhibited elevated levels of highly glycosylated EMMPRIN compared to BPH-1 cells. Panel **B**: LNCaP and PC-3 prostate cancer cells exhibited a significantly higher amount of membrane anchored EMMPRIN compared to BPH-1 cells. Membrane fractions (30 μg protein) were subjected to Western blotting. (**C**) EMMPRIN transcripts were analyzed by RT-PCR and electrophoresis. Splicing variants 2, 3, and 4 were confirmed by DNA sequencing as Band #3, #1, and #2, respectively.

The alternative splicing isoforms of EMMPRIN in the prostate cell lines were also determined. Four splicing isoforms of EMMPRIN have been deposited in the NCBI database and most studies focus on variant 2 that harbor two Ig domains. Two sets of primers were designed for RT-PCR: One starting at exon 1 and ending at exon 11, and the other one starting at

exon 2 and ending at exon 11. The RT-PCR products were analyzed by agarose electrophoresis and the results are shown in Figure 1 (Panel C). The RT-PCR products were cloned and subjected to DNA sequencing. The sequencing results demonstrated that there are three different splicing variants existed in human prostate cells: Variant 2 (band 3, 828 bp), variant 4 (band 1, 634 bp), and variant 3 (band 2, 793 bp). Other bands indicated by filled triangles were non-specific RT-PCR products. Variant 2 appeared to be the major transcript in human prostate cells and there were no evident differences in the splicing isoforms among the different cell lines.

EMMPRIN Silencing in PC-3 Prostate Cancer Cells

The functional significance of EMMPRIN in prostate cancer progression remains unknown. Thus we examined whether high levels of EMMPRIN in PC-3 cells, functionally contribute to the aggressive behavior of metastatic prostate cancer cells. Since PC-3 exhibits high endogenous EMMPRIN expression, we used the RNA interference approach to silence EMMPRIN in these cells. Three pairs of oligos targeting to EMMPRIN exon 5, 6, and 11 were designed and successfully cloned into pSUPER plasmid (containing GFP marker). Due to the low transfection efficiency in PC-3 cells (about 30% using FuGENE), cells with the GFP marker were sorted for 36 hr after transfection and were subjected to Western blot analysis. The results shown in Figure 2A indicate that EMMPRIN protein levels are significantly reduced by all three shRNA species. Stable clones in which EMMPRIN was silenced under G418 selection, had lower EMMPRIN levels compared to scramble controls (Fig. 2, panels A and B).The shRNA 277 clone, in which the middle region of EMMPRIN gene was targeted, had less of an effect in reducing EMMPRIN expression.

Effect of EMMPRIN Loss on Prostate Cancer Cell Proliferation and Apoptosis

To determine the role of EMMPRIN on prostate cancer cell growth, we initially examined the consequences of EMMPRIN silencing on prostate cancer cell proliferation, cell cycle, and apoptosis. Interestingly, down-regulation of EMMPRIN resulted only in a modest inhibitory effect on prostate cancer cell growth (Supplementary Fig. S1, panel A). Cell cycle analysis demonstrated no significant effect on cell cycle progression in shRNA EMMPRIN PC-3 transfectants (Fig. S1, panel B). Evaluation of apoptosis based on the TUNNEL assay revealed that loss of EMMPRIN had no significant consequences on the rate of cell death among these cell populations

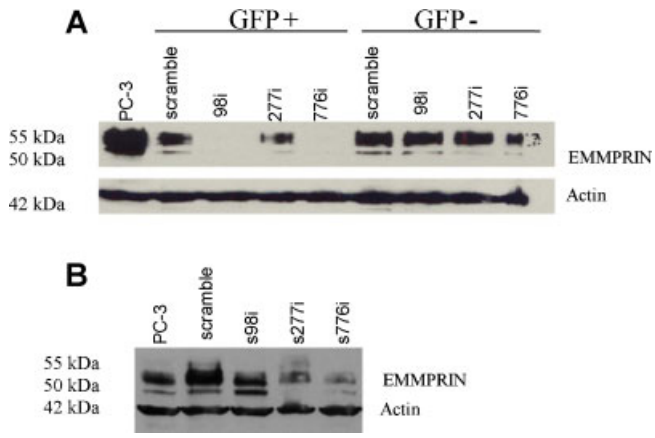

Fig. 2. Suppression of EMMPRIN expression in transient and stable shRNA prostate cancer transfected cells. PC-3 cells were transfected with three EMMPRIN shRNA plasmids or scrambled control and subjected to Western blot analysis to establish EMPPPRIN protein expression levels (major band detected at 50 kDa). Panel **A**: After transient transfection a considerable reduction in EMMPRIN expression was detected in GFP-positive cells transfected with three different shRNA plasmids, while no changes in EMMPRIN levels were observed in the GFP negative control cells. Panel **B**: Stably transfected cells with GFP marker were obtained after selection with G418 (300 μg/ml). Substantial reduction in EMMPRIN levels was demonstrated in the individual stable shRNA clones.

(Fig. S1, panel C). Thus, EMMPRIN is not involved in the control of prostate cancer cell growth or apoptosis.

EMMPRIN Loss Decreases Prostate Cancer Cell Adhesion, Migration, and Invasion

Many cell surface proteins are involved in cell adhesion and EMMPRIN can be a potential partner with such adhesion molecules. To determine the functional contribution of EMMPRIN to prostate cancer cell adhesion to the ECM, we examined attachment ability of EMMPRIN silenced PC-3 transfectants to key components of the ECM, fibronectin, and laminin. As shown in Figure 3 (Panel A), there was a 40% decrease in the number of cells attached to fibronectin for the EMMPRIN knockdown cells compared to the scramble control cells. A similar magnitude of suppression of cell adhesion to laminin was observed in the EMMPRIN shRNA stable clones compared to scramble control cells (Fig. 3, panel B) or PC-3 parental cells (approximately 30–50% suppression). We subsequently examined the consequences of EMMPRIN loss on prostate cancer cell migration. EMMPRIN silencing yielded a significant reduction in cell migration ability in all three shRNA prostate cancer cell lines (Fig. 3, panel B), with the s277i clone

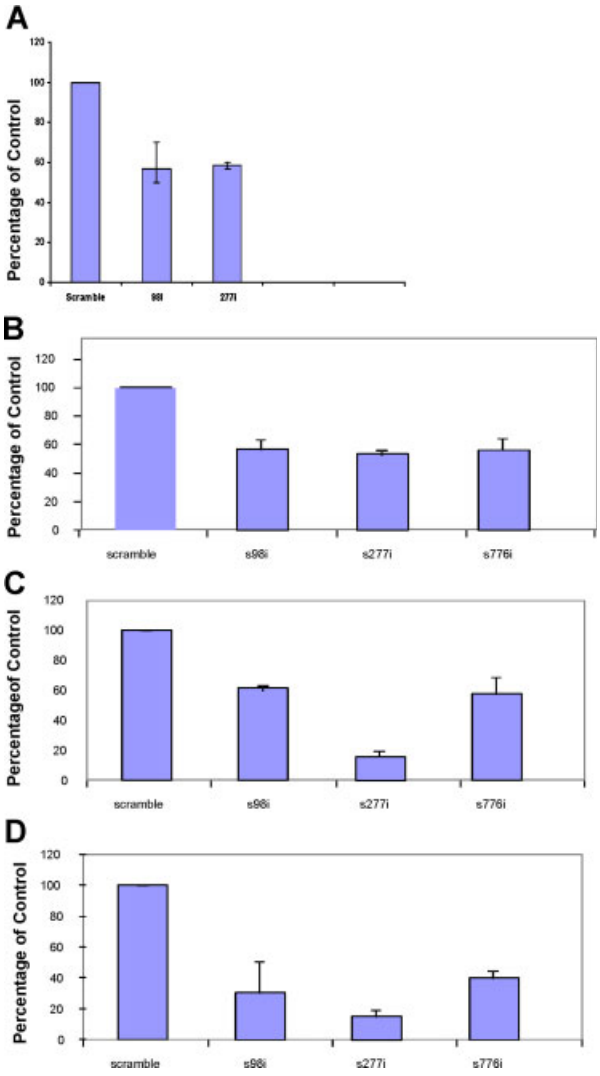

Fig. 3. Consequences of EMMPRIN silencing on prostate cancer cell adhesion to ECM, migration, and invasion. EMMPRIN shRNA transfectant PC-3 cells were seeded on fibronectin-coated (panel **A**) or laminin (panel **B**) plates for 30 min and attached cells were fixed and counted. Panel **C**: Cell migration was assessed by wounding the cell monolayer and determining the number of cells migrating to the wounded area after 24 hr. Panel **D**: The effect of EMMPRIN silencing on prostate cancer cell invasion was determined using the matrigel assay as described in "Materials and Methods" section. The average values from three independent experiments performed in triplicate are shown. Numerical values are expressed as percentage of control Sh scramble cells. Statistical significance is reached at $P < 0.01$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

exhibiting the most significant suppression. In addition, we examined the impact of EMMPRIN loss on the invasion ability of PC-3 cells. Figure 3 (Panel C) shows a significant decrease in cell invasion observed in EMMPRIN shRNA transfected cells compared to control cells. Thus, loss of EMMPRIN significantly decreased the adhesion, migration, and invasion abilities of metastatic prostate cancer cells.

EMMPRIN Enhanced Filopodia Formation in Prostate Cancer Cells

To determine whether EMMPRIN promotes cell migration by facilitating cytoskeleton reorganization, we examined the ability of EMMPRIN shRNA PC-3 cells, to form filopodia. Cells attached to fibronectin-coated cover-slips were subjected to immunofluorescence analysis for vinculin and F-actin presence and localization. The image in Figure 4 (Panel A), indicates that EMMPRIN silencing inhibited prostate cancer cell spreading on fibronectin, while a stronger F-actin staining was detected forming a stress fiber but without typical focal adhesion complex (Fig. 4, panel A). Confocal microscopy revealed a significant suppression of filopodia formation as a consequence of EMMPRIN loss. An approximate 50% reduction in the number of filopodia is detected in EMMPRIN knockout cells compared to control cells (Fig. 4, panel B). In addition, EMMPRIN silencing also led to a decrease in the strength of the filopodia. Immunofluorescence analysis (Fig. 4, panel A) revealed considerably larger filopodia in control cells compared to limited and small filopodia observed among EMMPRIN knockdown PC-3 prostate cancer cells.

Effect of EMMPRIN Knockdown on Cell Aggregation and Tight Junction Proteins

We subsequently examined the effect of EMMPRIN on the dissociation/detachment of cancer cells. A cell aggregation assay was conducted in the PC-3 control and EMMPRIN shRNA PC-3 prostate cancer cells. As shown in Figure 5 (Panel A), there was increased cell aggregation in EMMPRIN silenced PC-3 cells. Subsequent experiments determined the effect of EMMPRIN silencing on the expression of tight junction proteins. The levels of plasma membrane proteins JAM-A and JAM-B were unchanged in the EMMPRIN knockdown clones (Fig. 5, panel B). A significant increase however in the levels of tight junction associated proteins ZO-1, ZO-2, AF6, and β-catenin was detected consequential to EMMPRIN loss. These data imply that EMMPRIN may impair cell–cell interactions by facilitating the dissociation/detachment of tumor epithelial cells from each other.

DISCUSSION

To determine the cell surface protein differences between malignant and benign prostate cells and their significance in prostate cancer metastasis, we performed mass spectrometry analysis to profile the

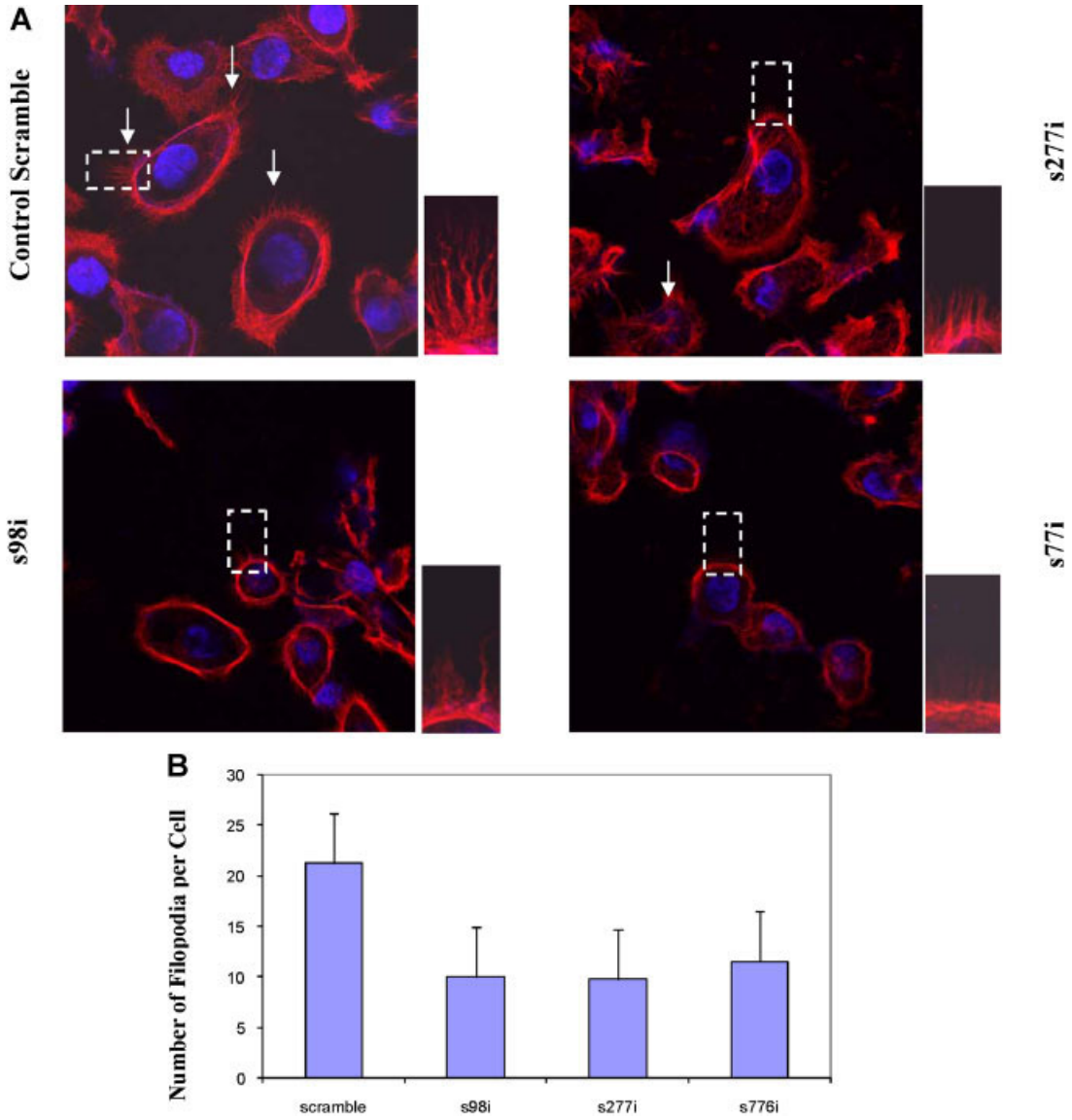

Fig. 4. EMMPRIN loss reduces filopodia formation in prostate cancer cells. Panel **A**: Cells were plated on fibronectin-coated glass coverslips and after spreading (24 hr), they were exposed to F-actin staining, and subsequently visualized under confocal microscopy; arrow heads indicate individual filopodia. The insert represents a zoom-in of region indicated in doted boxed areas. Panel **B**: The average number of filopodia in each cell was quantified in scramble control and the three shEMMPRIN clones, 98i, 277i, and 776i cells. Filopodia from at least 20 cells were counted and representative average values are shown. Approximately 20 cells/field and 10 random fields were examined for each cell line; error bars indicate average values from these measurements (mean) $\pm$ standard error of mean (SEM) gathered from three independent experiments. Statistical difference is considered significant at $P < 0.01$.

expression of cell surface proteins in human prostate cancer cells derived from metastatic lesions and benign prostate epithelial cells. One of the proteins highly expressed on the cell surface of metastatic prostate cancer cells, but not benign cells, was identified to be extracellular matrix metalloproteinase inducer (EMMPRIN, also known as basigin, CD147, OX47 or 5A11). EMMPRIN has been previously shown to be involved in cancer development via its ability to stimulate MMP production and consequently control extracellular matrix remodeling and anchor independent growth [28]. In addition, EMMPRIN has been shown to regulate angiogenesis by engaging the AKT–PIK3 pathway [19], and to up-regulate urokinase-type plasminogen activator [18]. EMMPRIN can also interact with key adhesion proteins such as integrins [23], implicating its role in cancer cell migration and invasion. The present study provides the first evidence on the functional consequences of EMMPRIN loss on prostate cancer cell growth, proliferation, apoptosis and cell adhesion (Fig. 3). We observed that down-regulation of

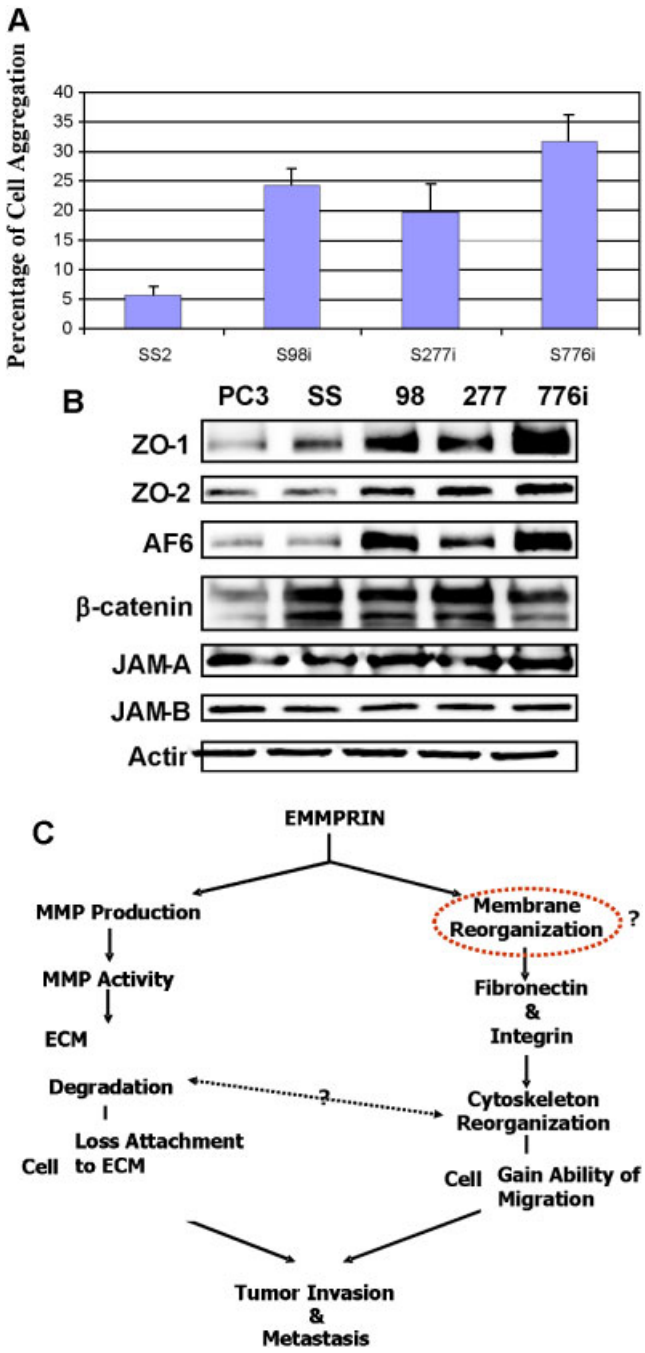

Fig. 5. Effect of EMMPRIN on cell aggregation and tight junction proteins. Panel **A**: Cell aggregation increased with reduced EMMPRIN expression in prostate cancer cell EMMPRIN Sh clones. Panel **B**: Western blotting indicating expression of gap junction proteins in EMMPRIN silenced PC-3 cells. Panel **C**: Potential role of EMMPRIN in prostate cancer metastasis. EMMPRIN can stimulate production of MMPs, leading to reduced cell adhesion to ECM. Alternatively, EMMPRIN may directly promote the metastatic potential of prostate cancer cells by enhancing migration and invasion through cytoskeleton reorganization and impairing cell–microenvironment interactions. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

EMMPRIN led to a significant suppression of prostate cancer attachment to fibronectin, a major ECM component (Fig. 4, panel A). Thus a defect in the cytoskeleton organization can be induced by functional loss of EMMPRIN. Furthermore down regulation of EMMPRIN protein led to decreased prostate cancer cell migration. Considering the evidence that cell migration is independent of MMPs and that MMP and EMMPRIN knockout mice [29] have different phenotypes, it is reasonable to postulate that these two proteins may operate in independent pathways functionally converging downstream. EMMPRIN may be engaged in distinct signaling pathways, directly promoting the invasive behavior of prostate cancer cells towards metastasis. This notion gains support from evidence indicating lack of correlation between EMMPRIN expression and MMP activity during adult mouse mammary gland development [30]. Moreover, EMMPRIN has been shown to directly promote insoluble fibronectin assembly [21].

In this study, EMMPRIN loss significantly reduced prostate cancer cell filopodia formation on a fibronectin substratum. This defective filopodia formation implies disruption of cytoskeleton organization and actin signaling in cells lacking EMMPRIN. These observations are consistent with reports suggesting that EMMPRIN (D-basigin in Drosophila) tightly regulates cytoskeleton rearrangement in *Drosophila melanogaster* [23]. Based on the present results and the existing evidence, we propose that EMMPRIN promotes tumor cell metastasis in an MMP-dependent and -independent pathway (Fig. 5, panel C). One must also consider that EMMPRIN has been associated with prominent membrane proteins caveolin-1 and vimentin, implicating its involvement in lipid raft and control of membrane dynamics. Here we show for the first time that silencing EMMPRIN resulted in enhanced cell aggregation (Fig. 5, panel A) and increased the protein expression for several tight-junctions mediators including ZO-1, ZO-2, AF-6, and β-catenin (Fig. 5, panel B). Considering the reported relationship between tight junction proteins and cytoskeletal changes associated with cell aggregation [27,31] our findings provide new insights into the ability of elevated EMMPRIN to navigate tight junctions and cell–cell adhesion within the tumor microenvironment. The mechanistic scenario discussed above can lead to enhanced prostate cancer invasiveness by EMMPRIN overexpression. Significantly enough, our group recently demonstrated that talin1, an actin-binding protein that links integrins to actin cytoskeleton in focal adhesion complexes, correlated with prostate cancer progression to metastasis [32]. Mechanistically, talin1 binding to β integrin recruits the focal adhesion partners ILK, FAK, and SRC, and

activates downstream signals, PI3K/Akt, and Erk; activation of this signaling promotes cell survival, migration and invasion, and resistance to anoikis. EMMPRIN may serve as an upstream partner for talin, facilitating its role in anoikis resistance and actin cytoskeleton remodeling, and consequently promoting metastatic spread.

Mammalian cells ubiquitously adopt a variant splicing strategy to cope with multiple functions and their requirement by diverse physiological processes. At least two different variants of EMMPRIN have been reported. Variant 2 is a ubiquitous expression protein as previously reported and a larger variant 1 is expressed in retinal epithelial cells in a tissue specific fashion. In this study, we identified three distinct EMMPRIN splicing variants: Variants 2, 3, and 4 (Fig. 1, panel C). The latter two variants are distinct from the commonly found variant 2. Significantly enough these two variants lack exon 2 where glycosylation occurs [3]. Moreover, variant 4 lacks exon 5, where another glycosylation site is also located. The dynamics of the ratio of different isoforms and the mechanisms via which the different splicing variants are engaged to navigate EMMPRIN expression and activity to meet the physiological demands of both ECM remodeling and cancer cell motility are currently being pursued.

The present results are of translational significance as functional exploitation of EMMPRIN in prostate cancer metastasis may lead to new approaches for impairing the metastatic process by (a) reversing the ability of tumor cells to resist anoikis (thus enhancing their sensitivity to anoikis-inducing agents); and (b) interfering with the tumor cell migration and adhesion to secondary sites. Ongoing studies focus on immunoprofiling EMMPRIN expression in human prostate specimens from patients with primary and metastatic tumors to determine the significance of EMMPRIN as a marker of progression to advanced castration-resistant disease.

In summary, our findings demonstrate that EMMPRIN loss has a major impact on cell membrane reorganization and spatial disruptions that significantly affect prostate tumor cell adhesion, migration, and invasion. The present work provides new insights into the function of EMMPRIN as a contributor to prostate cancer cell metastatic behavior and its potential value as a therapeutic target during tumor progression.

ACKNOWLEDGMENTS

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Appendix 2

Martin, S.K., Vaughan, T.B., Atkinson, T., **Zhu, H.** and **Kyprianou, N**. Prostate Cancer Biomarker Update*. Oncology Reports,* 28: 409-417*,* 2012.

Emerging biomarkers of prostate cancer (Review)

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Abstract. Prostate cancer progression involves activation of signaling pathways controlling cell proliferation, apoptosis, anoikis, angiogenesis and metastasis. The current PSA-based test for the diagnosis of prostate cancer lacks sensitivity and specificity, resulting in missed diagnoses and unnecessary biopsies. Intense research efforts to identify serum and tissue biomarkers will expand the opportunities to understand the functional activation of cancer-related pathways and consequently lead to molecular therapeutic targeting towards inhibition of tumor growth. Current literature describes multiple biomarkers that indicate the properties of prostate cancer including its presence, stage, metastatic potential and prognosis. Used singly, assays detecting these biomarkers have their respective shortcomings. Several recent studies evaluating the clinical utilization of multiple markers show promising results in improving prostate cancer profiling. This review discusses the current understanding of biomarker signature cluster-based approaches for the diagnosis and therapeutic response of prostate cancer derived from panels of biomarker tests that provide a selective molecular signature characteristic of the tumor. As these signatures are robustly defined and their pathways are exhaustively dissected, prostate cancer can be more accurately diagnosed, characterized, staged and targeted with inhibitory antitumor agents. The growing promise surrounding the recent evidence in identifying and utilizing such biomarker panels, will lead to improvement in cancer prognosis and management of the therapeutic response of prostate cancer patients.

Contents

1. Introduction: the prostate cancer prediction challenge

Prostate cancer is the second leading cause of cancer-related death in men (1). With the current enhanced understanding of the molecular mechanisms leading to advanced metastatic disease, several factors present challenging obstacles in developing successful therapeutic modalities and screening tools for cancer detection and treatment (2). Malignant prostate cells progress through a series of genetic and epigenetic changes leading to aberrant proliferation, angiogenesis, evasion of apoptosis, metastasis to secondary sites and androgen independence (3). These pro-oncogenic pathways and key signaling molecules are currently being examined at the molecular and cellular level; with the application of this powerful technology in individual tumors, one would expect identification of novel markers indicating specific tumor properties in individual patients. A characterization of such biomarkers on a personalized level of analysis is expected to greatly impact the way physicians detect early prostate cancer and intervene to impair its progression to advanced disease.

Prostate cancer is characterized by distinct pathological changes indicating uncontrolled growth and biochemical emergence to androgen-independence. Consistent elevations in total prostate specific antigen (tPSA) in the serum, as well as marked decrease in apoptosis and tissue differentiation, are key factors in the progression of prostate tumors to advanced disease. Rigorous research efforts focused on androgen-independence and the determination of alternate

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Abbreviations: PSA, prostate specific antigen; tPSA, total prostate specific antigen; AR, androgen receptor; BPH, benign prostate hyperplasia; KLK-4, kallikrein-4; Src-3, steroid receptor coactivator-3; Mcm5/7, minichromosome maintenance protein 5/7; EPCA, early prostate cancer antigen; TNF-α, tumor necrosis factor α; IL-6, interleukin 6; ECM, extracellular matrix; uPA, urokinase-type plasminogen activator; EZH2, enhancer of zeste homolog 2; PSCA, prostate specific stem cell antigen; AR, androgen receptor; FAS, fatty acid synthase; EMT, epithelial-mesenchymal transition; AMACR, α-methyl-co-racemase; GOLPH2, golgi protein H2; EN2, engrailed-2

Key words: prostate cancer, prostate specific antigen, biomarkers

androgen receptor (AR) pathways that cells employ to become androgen-independent are gradually adding to the current state of knowledge. With over 100 identified AR regulators, dozens of proposed androgen-independent receptor mutations, and several mechanisms of independence recognized including AR overexpression, local androgen production by the prostate and, proteolytic AR alteration to an androgen-independent isoform, a daunting task is evident (4).

In clinical practice, tPSA analysis has been the 'gold' standard in determining the presence and stage of prostate cancer. Accordingly, many clinicians recommend yearly serum tPSA and digital rectal examination (DRE) for men age 50 and older. As of 2009, the United States Preventative Services Task Force maintains that current evidence is insufficient to assess the balance of benefits and harms of screening for prostate cancer in men younger than 75 (I statement) and recommends against screening men age 75 and older (D statement) (5). PSA is a protein produced only by prostate tissue and would seem to be an ideal marker for prostatic disease. However, serum tPSA variability and its limited specificity to cancer are two properties currently limiting utility in prostate cancer screening and characterization (6). As varying serum tPSA values are found in patients with normal prostate function, benign prostate hyperplasia (BPH) and prostate cancer, high serum tPSA levels are not exclusive to the presence of prostate cancer (6) and this realization has drawn considerable controversy. Furthermore, while upward-trending tPSA values are often evident in individuals with progressive cancer, an absolute, linear relationship does not exist between serum tPSA and prostate cancer stage and metastasis (6). Despite finely orchestrated efforts in the clinical and translational setting, physicians and researchers have been unable to determine a standard tPSA level corresponding with precise disease staging, relying instead on tPSA cut-off values that vary among experts (7). In short, the path of disease progression results in a unique tPSA curve. This fact makes the tPSA test an indicator of predisposition to prostate abnormality rather than a definitive testing standard.

The challenge remains to define a firm biomarker level that definitively signals cancer initiation and/or progression to metastasis. Subsequently, other novel biomarkers are being studied for usefulness in diagnosing, staging and treating prostate cancer. Cancer is a disease of accumulating mutations causing uncontrolled cell growth with the contribution of epigenetic changes that can change the tumor phenotype. Uniformity is non-existant among each histologic cancer type and within each individual tumor. Thus, researchers have found other biomarkers associated with prostate cancer to be similarly variable as the disease progresses, limiting use in characterizing the disease (8). Examination of biomarker combination panels provides promise for early and precise prostate cancer diagnosis, and potential for the development of personalized treatments targeting the tumorigenic pathway defining individual tumors.

2. Serum biomarkers

Serum biomarkers are molecules produced by normal and abnormal cells. These molecules travel in blood plasma and are identified by serum assays. The most established and widely recognized serum biomarker for prostate cancer is total prostate-specific antigen (tPSA). PSA is a serine protease, also known as kallikrein 3, produced in an androgen-dependent manner by prostate ductal epithelial cells. PSA is generated by the healthy prostate at low levels, but can increase in association with cancer proliferation and prostatic disease progression (6). Currently, PSA is used to diagnose and stage prostate cancer, but has fallen under criticism with challenges to its sensitivity and specificity. In addition to PSA, several other prostate serum biomarkers have been studied (Table I) and their potential utilization is considered below.

PSA. PSA is found in both serum and tissue however, total serum concentration, or tPSA, is most often used. While there is positive correlation between PSA and cancer progression, the correlation is not always consistent. Several variations of PSA have been studied including free-to-total PSA ratio, PSA density, PSA velocity and PSA isoforms. Free-to-total PSA ratio (or %free PSA) has been shown to increase the specificity for cancer diagnosis in patients with intermediate (4-10 ng/ml) tPSA values (9). This result is due to total PSA production increasing at a greater rate than that of free PSA in cancer patients, resulting in lower %free PSA. PSA density measures the tPSA per prostate volume. It relies on the premise that prostate cancer releases PSA into patient serum in greater proportion than the expected increase related to hyperplasia, resulting in greater PSA density in patients with prostate cancer vs. BPH. Based on reported results the validity of this measure has been debated (10,11). PSA velocity assesses the rate of PSA change over time. BPH yields a linear increase and cancer eventually results in an exponential increase (12). Problems with PSA velocity include poor sensitivity when initial PSA values are <4 ng/ml. Finally, different isoforms of both free and protein-bound PSA have been examined. Overall, sub-classifying the protein-bound isoforms has provided some advantage in distinguishing BPH from cancer; however, the effect is not greater than using %free PSA (13). In contrast, studies of free isoforms including BPH-specific PSA (BPSA) and pro-PSA (an inactive PSA precursor) have shown increased detection of clinically relevant cancers in patients with 2-10 ng/ml PSA and improved ability to differentiate from BPH (14).

Kallikrein-4. KLK-4 is an androgen-dependent serine protease found in both serum and tumor tissue. Day *et al* demonstrated the elevated levels of anti-KLK-4 antibodies in sera of prostate cancer patients (15). Recently, KLK-4 has been implicated as a proliferative factor in prostate cancer cells and a potential mediator of the epithelial to mesenchymal transition. Ectopic expression of KLK-4 in prostate cancer cells increased the proliferation rate and motility of cells (16), while overexpression of KLK-4 resulted in a decrease of E-cadherin expression and increase of vimentin expression signaling, a potential EMT event (17). The trypsin-like activity of KLK-4 functions to activate pro-urokinase-type plasminogen into urokinase-type plasminogen activator (uPA), as discussed below (15). As more specific roles in prostate cancer development are elucidated for KLK-4, there is considerable promise that their ease of detection could effectively be utilized to diagnose and treat prostate cancer with a panel of other biomarkers.

Steroid receptor coactivator-3. Src-3 (p/CIP, AIB1, ACR, RAC3, TRAM-1) is a 160-kDa protein and member of the

Table I. Summary of Current Prostate Cancer Biomarkers.

Prostate Cancer Biomarkers in 2011	References
Serum biomarkers	
PSA	
tPSA	(6)
% free PSA	(9)
PSA Density	(10,11)
PSA Velocity	(12)
PSA isoforms	(13)
BPH specific PSA (BPSA)	(14)
Pro-PSA	(14)
Kallikrein-4 (KLK-4)	(15-17)
Steroid Receptor Co-Activator-3 (Src-3)	(16-19)
Minichromosome maintenance 5 protein (Mcm5)	(20,21)
E-cadherin	(22-25)
Early Prostate Cancer antigen	(26,27)
Interleukin 6 (IL-6) and Interleukin 6 Receptor (IL-6R)	(28-30)
Prostate Cancer Gene 3 (PCA3)	(31-33)
Tissue biomarkers	
Urokinase-type Plasminogen Activator (uPA)	(28,34)
Enhancer of Zeste Homolog 2 (EZH2)	(35-40)
Prostate Stem Cell Antigen (PSCA)	(41,42)
Androgen Receptor (AR)	(4,43-45)
Fatty Acid Synthase (FAS)	(46,47)
α-methyl-co-racemase (AMACR)	(48,49)
GOLPH2	(48,49)
Engrailed-2 (EN2)	(50)

Src family (16). Src-3 is a non-receptor tyrosine kinase which possesses an innate histone acetyltransferase activity as well as acting as a scaffold for recruitment of other coactivators to the transcription initiation complex (17). The recruitment of Src-3 to the PSA promoter in the presence of androgen and the physical interaction between the steroid receptor and Src-3 have been implicated in tumorigenesis (16,18). However, Src-3 overexpression is not unique to hormone-dependent cancers although it is well characterized in cancers of the breast, ovary, and prostate. Src-3 overexpression has been observed in gastric and pancreatic cancer, which suggests it may be facilitating tumorigenesis via other transcription factor interaction partners (16). The increased presence of Src-3 in serum samples has been correlated with enhanced cell proliferation and hormone-independence and inversely-related to cell apoptosis (17). In patients undergoing radical prostatectomy, PSA recurrence is an indicator of metastasis and disease progression; patients which scored higher on Src-3 overexpression were significantly more likely to undergo recurrence (16). Therefore, Src-3 serves as a viable indicator for disease recurrence. The ability of Src-3 inhibitors to impair prostate cancer progression and metastatic spread is currently being evaluated *in vivo*. Interestingly, a population based study of the polymorphic CAG/CAA repeat length in Src-3 gene has provided some preliminary evidence that a racial-associated prostate cancer risk may lie herein (19). Src-3 may hold the potential to serve as both a risk determinant and an indicator of recurrence.

Minichromosome maintenance protein (Mcm5/7). Minichromosome maintenance proteins are key players in the initiation of DNA replication and chromosomal duplication (20). Interestingly they are expressed in all phases of the cell division cycle, but silenced in phases in which growth is not occurring. With respect to prostate cancer, it has been shown that Mcm5 is overexpressed in prostate tissue and serves as an independent predictor of survival in patients undergoing radical prostatectomy, androgen deprivation therapy or radiotherapy (21). Contributing further to its potential as a biomarker, is evidence of the low levels of Mcm expression in normal and benign hyperplastic prostatic tissue. Recent work by Dudderidge *et al* revealed that Mcm5 levels are increased in urine sediments of patients with prostate cancer compared to those without and confirmed that Mcm5 levels are not increased in patients with BPH (21). While Mcm5's role in prostate cancer detection and diagnosis is still currently being investigated, its usefulness on the development of a panel of biomarkers could be vital for the early detection of prostate cancer in the near future.

Mcm7 is another member of the proteins which together form a portion of the pre-replication complex which licenses DNA replication and is being investigated for its usefulness in identifying prostate cancer progression. An investigative comparison of Ki67 vs. Mcm7 immunohistochemistry staining was conducted and demonstrated that Mcm7 correlated highly with Ki67, but demonstrated an improved ability to distinguish between benign, PIN and adenocarcinoma (20). Further evaluation of Mcm7 expression with cancer progression, may prove the utility of this new marker.

E-cadherin. E-cadherin is a major mediator of cell-cell adhesion junctions insuring communication between neighboring healthy cells and their connection to the surrounding extracellular matrix (ECM). Anoikis is a unique mode of programmed cell death consequential to loss of adhesion to neighboring cells and the ECM (22). The ability of prostate cancer cells to evade anoikis, and thus successfully invade and metastasize is driven by loss of E-cadherin expression and upregulation of epithelial-mesenchymal transition (EMT) regulators (22). Elevated levels of serum cleaved E-cadherin were demonstrated in metastatic prostate cancer cells, conferring the loss of the need for adherence to the surrounding ECM matrix and tissue (23). Furthermore, evidence has pointed to the switching of cadherin type expression with cancer progression. The loss of E-cadherin expression and gain of N-cadherin and cadherin-11 expression is seen in epithelial derived tumors (24). This cadherin switching has been associated with enhanced invasive capacity, metastasis, and dismal clinical outcomes; furthermore, it may serve as a pivotal biomarker of epithelial to mesenchymal transition.

Further evidence of this molecule's therapeutic promise has been the recent use of small activating RNAs (saRNA) or non-coding, double stranded RNA molecules that can induce gene transcription by targeting promoter regions specific to the gene of interest. Through the use of saRNAs targeting E-cadherin expression, Mao *et al* demonstrated decreased cell migration and invasion of PC3 prostate cancer cells transfected with the E-cadherin specific saRNA (25). Thus, not only could the cleaved E-cadherin fragment be utilized as a promising marker of disease progression and metastasis, but it potentially could be targeted as an inhibitor of metastasis.

Early prostate cancer antigen (EPCA-2). Utilization of proteomics approaches has expedited the search for new biomarkers in cancer. Investigation into changes within the structural nuclear proteins have yielded identification of novel prostate cancer biomarkers (26). This characterized protein, EPCA-2, is elevated in sera of prostate cancer patients, but not in healthy patients. Subsequent studies have focused on raising antibodies against specific EPCA-2 epitopes that are both easily analyzed in serum, and specific to prostate cancer. The goal of these studies was to determine an effective screening tool for prostate cancer. One epitope, EPCA-2.19 shows considerably promise (27). An initial study from known samples determined a serum cut-off value of 0.5 ng/ml EPCA-2. A follow-up prospective study of 328 men showed that EPCA-2.19 has 94% specificity and 91% sensitivity in separating normal men and men with BPH from those with prostate cancer using the aforementioned cut-off of 0.5 ng/ml (27). Antibodies against another epitope of the same protein, EPCA-2.22, have furthermore been shown to distinguish organ-confined from non-organ-confined prostate cancer. One could envision how combination assays including both antigens might be applied for detection and staging of prostate cancer (27).

Interleukin-6 (IL-6) and interleukin-6 receptors (IL-6R). The cytokine interleukin-6 is most commonly known for its role in inflammation but has recently been evidenced for a role in the development of different cancers including prostate cancer. Elevated IL-6 and its soluble receptor have been linked to aggressive prostate cancer features including increased tumor volume, elevated overall Gleason score, distant metastases and decreased survival (28). *In vivo* studies have suggested a pathogenic role for the cytokine in prostate cancer and thus sparked new research involving the mechanisms of its effect (29). In order to exert its cellular effects, IL-6 must bind to the IL-6 receptor (IL-6R) to form a complex capable of binding to specific signal transducing proteins on the cell membrane. Two forms of the receptor exist, one being membrane bound (mIL-6R) and the other being soluble (sIL-6R). The soluble receptor isoform has been implicated as a predictor of metastatic disease. Its elevation along with the elevation of IL-6 has been demonstrated in patients who develop metastatic disease vs. patients who do not have disease recurrence within 5 years (29). It was further shown that sIL-6R compared to IL-6 demonstated a more robust correlation with disease progression (28). Recent work by Santer *et al* demonstrated increased cell motility and migration as well as decreased cell adhesion of prostate cancer cells in the presence of IL-6 with sIL-6R, but not IL-6 alone (30). Further understanding of the IL-6 pathway and the effect of its soluble receptor bound form will allow for more specific utilization of IL-6 as a marker of prostate cancer progression and metastasis. The incorporation of IL-6 and sIL-6R into a panel of preoperative blood based biomarkers improved the predictive capacity of the panel significantly in patients undergoing radical prostatectomy (28).

Prostate cancer gene 3 (PCA3). PCA3 is a prostate specific non-coding RNA which has been found to be highly overexpressed in >95% of primary prostate tumors, and furthermore, a 66-fold upregulation compared to adjacent non-cancer tissues (31). Through the intense contributions by Jack Schalken's group, the recognition of this attractive new marker for prostate cancer shows considerable promise. Thus, the prostate cancer gene 3 (PCA3) assay has been argued in European and USA studies to better identify men at high risk of a positive biopsy and moreover to discriminate the best candidates for a repeat biopsy. Significantly enough, the probability of a positive repeat biopsy increased with increasing PCA3 score (31). In the clinical setting, the best diagnostic accuracy is potentially obtained in the 'grey' zone in which the yield of the free-to-total PSA ratio (f/tPSA) is maximal. Testing for elevated PCA3 has evolved into a quantitative urine test to facilitate prostate cancer diagnosis via non-invasive methodology (32). Comparison of the performance of PSA vs. PCA3 urine test was investigated by Roobol *et al* (33). They found that as a first line screening, PCA3 was an improved evaluative tool for performance characteristics and identification of serious

disease in a prescreen population of 721 men (33). Based on the development of the clinical urine test and incorporation into many prostate cancer risk calculations, PCA3 is likely to become a lead biomarker.

3. Tissue biomarkers

Studies utilizing tissue specimens taken during diagnostic biopsy or radical prostatectomy, have shown that the expression of certain proteins, including uPA, enhancer of zeste homolog 2 (EZH2), prostate specific stem cell antigen (PSCA), androgen receptor (AR) and fatty acid synthase (FAS) correlates with tumor stage. The value of these molecules in the clinical arena is not limited to diagnosis, but many of these biomarkers produced could potentially be targeted to disrupt tumor progression to metastatic sites. The following tissue markers are currently being investigated for their clinical value in prostate cancer.

Urokinase-type plasminogen activator. The activation of the uPA cascade via the interaction of the inactive precursor with a soluble or membrane bound uPA receptor (uPAR), results in extracellular matrix remodeling via degradation of the ECM and the basement membrane. The activation of a broad spectrum proteases by the uPA network facilitates metastasis of tumor cells and angiogenesis (28). Amplification of the *uPA* gene and increased *uPA* copy number have been described in patients with metastatic prostate cancer (34). This evidence supports a potential role for uPA as a molecular target for both early identification and inhibition of metastatic prostate cancer. Reported results established that uPA inhibition leads to a marked reduction in the invasive ability of prostate cancer cells (34). As described for IL-6/sIL-6R, the inclusion of uPA level in a preoperative blood based panel of biomarkers significantly enhanced the predictive power of the panel (28).

Enhancer of zeste homolog 2. The pioneering work by Chinnaiyan's group identified EZH2 protein in many human malignancies, including renal, breast and prostate cancer (35). The expression of this protein is associated with cancer metastases, localized to tumors with poor prognosis and found in combination with depressed E-cadherin expression and associated short term disease-free survival (36). EZH2 functions as a histone methyltransferase and its overexpression has been evidenced in castration-resistant, metastatic prostate cancer. Analysis by Li *et al* found that levels of EZH2 RNA and protein were significantly higher in prostate cancer cells than BPH or in human prostate intraepithelial neoplasia (HGPIN) (37). Also there was a significant increase in EZH2 in tumors with aGleason score >7 vs. <6 in addition to the significant positive correlation of EZH2 to TNM stage increasing with tumor progression (37). Further study into specific mechanisms of action of EZH2 have linked it with the gene fusion found in 50% of prostate cancers of TMPRSS2, an androgen-regulated gene, and the oncogenic ETS transcription factor ERG. ERG itself activates EZH2 allowing the methyltransferase to induce its repressive epigenetic agenda (38). The neuronal chemorepellant and tumor supressor gene SLIT2 has also been linked to EZH2. EZH2 targets SLIT2 and inhibits its expression (39). Levels of SLIT2 have been found to be downregulated in a majority of prostate cancers and a low level of SLIT2 has been associated with not only agressive prostate cancers, but breast and lung cancer as well (39). SLIT2 is downregulated via hypermethylation of the SLIT2 promoter accomplished by the enzymatically catalyzed actions of EZH2 on the lysine 27 of histone H3 (39).

In vitro studies have successfully shown the inhibition of prostate cancer cell proliferation using molecules targeting EZH2 (35). Recently, microRNA technology was effectively used to inhibit EZH2 expression resulting in a decreased migratory and invasive ability of prostate cancer cells (40). Although this pattern of overexpressed EZH2 and depressed SLIT2 is observed in other cancer types, the combination of these two may serve as a pertinent duo-panel of characteristic biomarkers for prostate cancer prognosis. Additional trials will enable the documentation of an association between elevated EZH2 with prostate tumor aggressiveness and low SLIT2 expression linked to poor prognosis (39).

Prostate stem cell antigen (PSCA). PSCA is a unique antigen found in prostate tissue, both healthy and diseased. In a recent study, PSCA was expressed in 94% of primary tumors and 100% of metastatic samples (41). Higher levels of PSCA were also significant in predicting an increase in cancer stage, Gleason score and androgen-independence. In another study, PSCA mRNA expression in tissue acquired from transurethral resection of the prostate (TURP) for BPH in patients with negative preoperative biopsy predicted subsequent cancer incidence (42). Therefore, PSCA serves as another potential therapeutic molecular target, as well as prognosticator of cancer incidence and progression.

Androgen receptor (AR). AR is a key protein functioning as a nuclear transcription factor in prostate cancer cells that may be used on a panel for prostate cancer screening. Prostate cancer progression is associated with acquisition of androgen-independence, resulting in metastatic lesions (4). Indeed, the emerging understanding of the mechanism of therapeutic failure of advanced prostate tumors involves upregulation of AR or activation of its transcriptional activity via a ligand-independent manner leading to castration-independent disease (43). Mechanisms of this development have been proposed including the development of an AR splice variant. Studies by Sun *et al* demonstrated a particular splice variant of AR found in humans whose transfection into mice led to castration-resistant prostate tumors and whose ratio to the full length androgen receptor positively correlated with castration resistant disease (44).

The relationship of the AR and its functionality to currently researched prostate cancer biomarkers is an area resounding with promise. A study by Dahlman *et al* studying the prostate cancer marker β-microseminoprotein (MSMB) has shown that its expression is in part controlled by androgen availability and a low level is associated with poor outcome and more aggressive disease (45). Further support for its inclusion in a biomarker panel is offered by the fact that MSMB expression was shown to be associated to high EZH2 expression and thus could be a possible target of epigenetic silencing effects (45). Consequently, the AR may serve not only as a therapeutic target, but also as a candidate for biomarker panels predicting prostate cancer metastasis, independent of androgens.

Fatty acid synthase (FAS). FAS is an androgen-regulated metabolic enzyme involved in *de novo* biosynthesis of fatty acids (46). *FAS* mRNA and FAS protein are both significantly overexpressed in prostate carcinomas (47). Furthermore, mRNA and protein levels were demonstrated to increase progressively with normal prostate to prostatic intraepithelial neoplasia, low grade, high grade and androgen-independent bone metastases (47). Based on these observations, FAS expression may be useful as a biomarker to assess disease staging and progression, especially because elevated FAS expression is seen in all neoplastic tissues. Moreover, molecular profiling studies by Swinnen *et al* suggest that this biomarker is not only one of the earliest appearing but also one of the most common molecular alterations in prostate cancer (46).

α-methyl-co-racemase (AMACR). AMACR is an emerging biomarker which has already achieved clinical acceptance. This protein has been utilized in combination with other cancer markers to visualize infiltration of prostate cancer glands into negative benign prostatic parenchyma facilitating diagnosis (48). AMACR has been utilized in a panel of biomarkers including ERG, GOLPH2 and others to definitively detect early prostate cancer (49).

GOLPH2. GOLPH2 is a 73-kDa Golgi phosphoprotein of unknown function which has been characterized as a biomarker of prostate cancer (48). Elevated mRNA expression is upregulated in prostate cancer specimens and has been shown to provide greater predictive capacity than PSA (49). Overexpression of GOLPH2 protein has been histologically demonstrated as well, but detection is slightly less sensitive than that of AMACR (48). Laxman *et al* have demonstrated that through the use of urine sedimentation and qPCR early detection of prostate cancer can be determined with greater accuracy than the PSA blood test and >75% positive predictive value. GOLPH2 was incorporated into the multiplex biomarker panel used therein (49).

Engrailed-2 (EN2). A subset of genes involved in early embryonic development have been shown to be reawakened during cancer development, notably the *HOX* genes. EN2 is a member of this gene family and has been identified as a transcriptional repressor as well as a translational regulator (50). Investigation into the activation of this gene has yielded a tumor specific biomarker which is secreted by prostate cancer tissue and can be detected in first pass urine (50). Elevated EN2 expression was identified in conditioned media from the prostate cancer cell lines PC3, DU145 and LNCaP, and confirmed in patient biopsies. Development of an ELISA test for detection of EN2 in urine is underway and holds promissing predictive capabilities if confirmed via further investigations.

4. Molecular signatures

Just as a fingerprint is unique to each person, cancer cell lines exhibit signature protein pathways, differentiating them from surrounding tissues and other tumors. When these molecular signatures are determined, an individual cancer can be definitively identified, assigned an expected pattern of disease progression, and therapeutically targeted. In theory, this will increase diagnostic accuracy and prolong patient survival. Surprisingly, little research has been invested in examining panels of known prostate cancer biomarkers and their utility. Using existing knowledge, the discovery of novel molecular tumor signatures will enable researchers to diagnose and stage cancer accurately, while opening up a field of selective therapeutics.

TMPRSS2:ETS gene fusions. The TMPRSS2:ERG chromosomal rearrangement identified by Chinnayan's pioneering studies in 2005 has become a molecular event of historic proportions in the prostate cancer field. The androgen regulated transmembrane serine protease TMPRSS2 is secreted by prostate epithelial cells in response to ligand exposure and this gene becomes fused with sequences of members of the ETS family of transcriptional activators (ERG, ETV1,4,5). Since TMPRSS2 is expressed in the prostate and regulated by androgens, its fusion to the transcriptional activators ETS gene products could result in driving prostate cancer development, and it appears that this is in fact the case. The prevalence of the fusion products seems to be quite high, although reports vary given that modes of detection vary, different fusion species may exist in a single tumor specimen, and new fusion rearrangements are still being discovered. Regardless, it has been reported that ≥70% of all prostate cancers possess a fusion product (51-57). Despite the functional validation of the prevalence of the fusions, the prognostic value in the clinical setting of prostate cancer patients is still under pursuit. Demichelis and colleagues investigated the impact of the fusion in a watchful waiting cohort of 111 patients and found that those with the fusion had a 2.7-fold increase in prostate cancer-specific mortality compared to those without the fusion, and after 8 years 23% of those without the fusion progressed to metastatic prostate cancer (58). Extensive investigation remains in order to understand the TMPRSS2:ERG gene fusion products role in prostate cancer progression, but it is clear that this molecular event is an early and important marker of prostate cancer. Other groups further investigated the molecular implications of the gene fusion and attempted to identify other genetic prognostic markers in order to develop a panel of genetic signatures that would provide prognostic prediction of biochemical recurrence, based on a cohort of specimens used previously to characterize expression of TMPRSS2:ERG variants (54,59). Using cDNA-mediated annealing selection extension and ligation assay (DASL), 9 upregulated (ERG, HDAC1, ARHGDIB, TRAF4, MSH3, MUC1, YES1, ING1, E2F3) and 6 downregulated genes (CD44, IGF1, MAF, IGFBP6, PTGS1, FZD7) were identified in TMPRSS2:ERG fusion-positive tumors from the aforementioned cohort of samples (59). Using gene ontology analysis, it was determined that mismatch base repair and histone deacetylation functions were over-represented in those genes upregulated with the fusion, and insulin-like growth factor and Jak-Stat signaling pathways in the downregulated genes. These data suggest that there is a unique molecular metabolism functioning in TMPRSS2:ERG fusion-positive tumors. Furthermore, replicating the analysis in a second cohort, Barwick *et al* (59) delineated a set of 9 genes associated with recurrence (CSPG2, CDKN2A, WNT10B, TYMS, E2F3) and

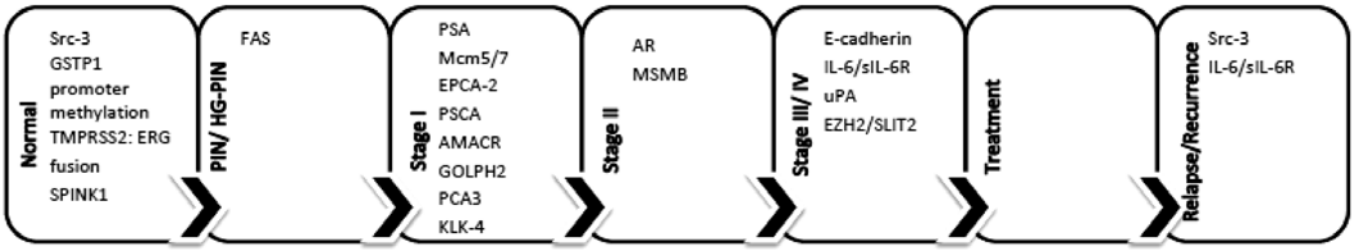

Figure 1. Emerging biomarkers of prostate cancer: continuum of disease progression and stage at which respective biomarkers are anticipated to facilitate clinical utility.

non-recurrence (TGFB3, CD44, ALOX12, LAF4) in these patient samples. From these efforts, it was demonstrated that TMPRSS2:ERG fusion status provides a statistically significant predictor of recurrence (P=0.0004), and that the 9 gene panel also yielded a statistically significant predictor of recurrence (P<0.03) (59). The investigation of these clinical and molecular factors provided a molecular signature platform for predicting recurrence in prostate cancer.

Serine protease inhibitor Kazal type 1 (SPINK1). SPINK1 is emerging as a biomarker of a molecular subtype of prostate cancer, in the absence of gene rearrangements/fusions such as TMPRSS2:ERG (60). Although long recognized in pancreatic physiology, SPINK1 more recently reemerged as an independent prognostic marker for a variety of cancers, but lacked superiority in predictive value to other commonly used markers (60). Tumors which produce SPINK1 also co-produce activated trypsin. It has been demonstrated that ~10% of prostate cancer cases are SPINK1^{+}/fusion^{-} and that this profile can be detected via non-invasive urine assays (61). Furthermore, the SPINK1^{+} outlier expression is a positive predictor of biochemical recurrence after resection, of an aggressive phenotype, and is correlated with Gleason score and poor prognosis (60-62).

New evidence has identified FAS expression as a molecular signature of highly aggressive metastatic prostate cancer. Rossi *et al* characterized unique gene expression profiles that differed significantly between prostate tumors expressing low and high levels of FAS. Increasing FAS protein expression directly correlates with cancer progression (47). In another study, 4 genes, *XLKD1*, *CGA*, *F2R* and *BCL-G*, have been found to reveal cancer recurrence in patients, independent of disease progression (44). An independent analysis of 16 related biomarkers revealed a functional connection with the tumor grade, with each molecular biomarker being assigned to one of five groups based on function. Significantly, it was found that specific composite score for these markers correlated with the Gleason score and cancer staging (63). Moreover, this study firmly established that the composite score was of higher predictive value of cancer grade and relapse than any one of the marker levels alone, confirming the hypothesis that a panel of biomarkers can be more effective for cancer diagnosis than a single marker (63).

Promising advances in biomarker determination have centered around DNA promoter hypermethylation with regard to SLIT2 (64). This epigenetic alteration, well-characterized in a variety of cancer cells, consistently trends toward conserved promoter regions. Gene panels have been explored with fine tuning and those of interest include genes involved in DNA damage and repair, tumor suppressor gene activation, hormonal responses, cell cycle checkpoints and invasion/metastasis. By combining different genes into one panel testing for hyper-methylation, the sensitivity of the panel can be exquisitely enhanced and ultimately refining the discriminatory power of the panel for the exclusive selection of prostate cancer diagnosis. Panels being used currently include combinations of the following genes: *GSTP1* (over-represented in panel compositions), *RASSF1a*, *RARβ*, *APC*, *PTGS2*, *T1G1*, *EDNRB* and *CDH13*, *ASC* (64). This method of hypermethylation detection of genes of interest shows tremendous promise and ability to predict prostate cancer presence and in some cases prognosis and relapse. *GSTP1* methylation has been evaluated independently as a biomarker of prostate cancer n urine sediments, revealing its ability to differentiate between BPH and prostate cancer; furthermore, the frequency of high methylation status correlates strongly with stage III and IV disease (65). The only limitation however for moving such a technique to the clinical setting towards detection of prostate cancer is the small sample sizes analyzed in these studies. Expansion in a considerably larger sample size may establish this technique as a relatively simple and sensitive method to detect prostate cancer.

Genome-wide association studies have implicated a handful full of single nucleotide polymorphisms with predisposition to prostate cancer development, specifically in the MSMB gene, which codes for β-microseminoprotein (66). This protein has previously been reported as an early serum biomarker for prostate cancer, but recent refinement has demonstrated that SNPs in the MSMB gene represents a predisposition factor for metastatic prostate cancer (66,67). Advancements of this nature may facilitate our ability to predict the course of disease progression in patients and more aggressively provide treatment based on these prognostic markers. Such studies represent few of the seemingly endless avenues, exploration of which could potentially uncover new diagnostic tools and implement clinical applications. A combination of biomarkers known to indicate prostate cancer proliferation, cell cycle progression, apoptosis loss and signaling of metastasis (anoikis resistance) could be made into a panel of indicative molecules that will present a novel platform of high predictive power of prostate cancer cases. A preliminary panel of emerging biomarkers based on prostate cancer stage is shown in Fig. 1.

5. Technology-driven new leads

Recent research has focused on mapping molecular pathways, but the future of prostate cancer research needs to progress in order to use of biomarker panels to detect and characterize tumors. Application of this data can be used to develop novel, tumor-specific treatments, focusing on implementing biomarker panels that will potentially predict the value of interfering with these proteins and their downstream signaling pathways. Information flow within and between cell and tissue compartments through a complex web of biochemical processes provides the ideal forum for biomarker identification. The most prominent features include post-translational modifications of newly synthesized proteins; interactions between kinases and non-enzymatic proteins such as adapters and scaffolds; sequestration within specialized subcellular compartments; intracellular transport; regulated secretion into extracellular space; and assembly, stabilization and disassembly of large, multimeric signaling complexes via ubiquitination and proteasome-mediated degradation. Most of these events are capable of providing critical regulatory control over cell growth, cell survival and apoptosis, anoikis and detachment from the ECM as well as interactions with adjacent cells, neovascularization/angiogenesis and membrane structure and trafficking.

The enormous gaps in our knowledge with respect to clinical pathologies seen in human disease remain. Considering high throughput proteomics data obtained from any physiologic or pathophysiologic situation, one may find that many of the true 'hits' have not been previously described at the protein level in any context. In addition, factors in signal transduction mechanisms exhibit a high degree of context-dependence and tissue specificity. Cleverly designed proteomics-based applications in human tissue microarrays from treated and untreated prostate cancer patients, will determine the genes that code for prostate cancer promotion and confer cancer cell survival and resistance to apoptosis and anoikis. Enhanced understanding of the complexity of the molecular mechanisms and expansion of investigative efforts driven by the sophisticated cutting-edge functional genomic and proteomics technology, may result in earlier detection of prostate cancer and more precise staging, and may offer a more accurate prediction and effective management, ultimately resulting in a strong and beneficial impact on patient survival.

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